AVIAN DISEASES 46:412–422, 2002

Experimental Infection of Turkeys with Avian Pneumovirus and Either Newcastle Disease Virus or Escherichia coli

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Received 24 September 2001

SUMMARY. Avian pneumoviruses (APVs) are RNA viruses responsible for upper respiratory disease in poultry. Experimental infections are typically less severe than those observed in field cases. Previous studies with APV and *Escherichia coli* suggest this discrepancy is due to secondary agents. Field observations indicate APV infections are more severe with concurrent infection by Newcastle disease virus (NDV). In the current study, we examined the role of lentogenic NDV in the APV disease process. Two-week-old commercial turkey poults were infected with the Colorado strain of APV. Three days later, these poults received an additional inoculation of either NDV or *E. coli.* Dual infection of APV with either NDV or *E. coli* resulted in increased morbidity rates, with poults receiving APV/NDV having the highest morbidity rates and displaying lesions of swollen infraorbital sinuses. These lesions were not present in the single APV, NDV, or *E. coli* groups. These results demonstrate that coinfection with APV and NDV can result in clinical signs and lesions similar to those in field outbreaks of APV.

RESUMEN. Infección experimental en pavos por pneumovirus aviar y el virus de la enfermedad de Newcastle o Escherichia coli.

El pneumovirus aviar es un virus RNA responsable de una enfermedad respiratoria del tracto superior de las aves. Las infecciones experimentales son por lo general menos severas que las observadas en el campo. Estudios realizados con anterioridad con el pneumovirus aviar y *Escherichia coli* sugieren que las diferencias observadas pueden ser debidas a la presencia de agentes secundarios. Observaciones de campo indican que las infecciones por pneumovirus aviar son más severas cuando se encuentran asociadas con el virus de la enfermedad de Newcastle. Se examinó el papel del virus lentogénico de la enfermedad de Newcastle en el curso de la enfermedad ocasionada por el pneumovirus aviar. Se infectaron pavitos comerciales de 2 semanas de edad con la cepa Colorado del pneumovirus aviar y 3 días después, estos pavitos fueron inoculados con el virus de la enfermedad de Newcastle o con *E. coli.* Se observó un aumento en el índice de morbilidad en infecciones mixtas del pneumovirus aviar con el virus de la enfermedad de Newcastle o con *E. coli.* El mayor índice de morbilidad se observó en pavitos que recibieron el pneumovirus aviar y el virus de la enfermedad de Newcastle, presentando inflamación de los senos infraorbitales. No se observó inflamación de los senos infraorbitales en los grupos que recibieron únicamente el pneumovirus aviar, el virus de la enfermedad de Newcastle o el *E. coli.* Las infecciones mixtas del pneumovirus aviar y del virus de la enfermedad de Newcastle pueden resultar en signos clínicos y lesiones similares a las observadas en epidemias de pneumovirus aviar en el campo.

Key words: avian pneumovirus, turkey rhinotracheitis, turkey, RT-PCR, Newcastle disease virus, *Escherichia coli*

Abbreviations: APV = avian pneumovirus; CFU = colony-forming units; CPE = cytopathic effect; DPI = days postinoculation; EID_{50} = 50% embryo infective dose; ELISA = enzyme-linked immunosorbent assay; HA = hemagglutination; HI = hemagglutination in-

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hibition; IBV = infectious bronchitis virus; Ig = immunoglobulin; i.n. = intranasal; NDV = Newcastle disease virus; OD = optical density; PBS = phosphate-buffered saline; PCR = polymerase chain reaction; RT = reverse transcriptase; $TCID_{50}$ = 50% tissue culture infective dose; TRT = turkey rhinotracheitis

Avian pneumoviruses (APVs) are negative-sense single-stranded RNA viruses that belong to the family *Paramyxoviridae* and the genus *Metapneumovirinae* (34). APVs cause turkey rhinotracheitis (TRT), a respiratory disease in turkeys, and have been associated with swollen head syndrome in chickens (12). The disease TRT was first described in South Africa during the 1970s (9). In 1986, APVs were isolated from clinically affected turkeys (29,44). Today, APV is found in South Africa, Japan, Europe, South and Central America, and the United States (3,12,24,40,41).

APVs have been tentatively designated as type A, B, C, or D on the basis of virus neutralization and sequence analysis (7). Type A and B viruses currently are found in Europe, Japan, and South and Central America and generally share approximately 83% amino acid sequence similarities in their fusion and matrix proteins (38,39). APVs isolated in the United States to date are type C viruses. Type C viruses are 78% similar to type A and 71% similar to B viruses with the fusion protein for comparison (39). Type D viruses, isolated in 1985, were recently characterized and found to be 70%–80.5% similar to type A and B viruses and 77.6%–97.2% similar to type C viruses (6).

Experimentally, APV is able to induce respiratory disease in turkeys, with clinical signs and lesions that include sneezing, depression, and rhinotracheitis with nasal exudates (12). More severe clinical signs of infection, coughing, head shaking, swollen sinuses, and increased morbidity and mortality, have been reported with natural infections. As with many infections, secondary agents and/or poor husbandry will prolong the disease and result in increased morbidity and mortality (12).

Dual infections with various bacteria and viruses have been shown to induce more severe clinical signs of infection. *Escherichia coli* can worsen the effects of APV infection in both chickens and turkeys (33,42). Clinical signs, including nasal exudates and frothy discharge from the eyes, are most severe when bacteria are given within 3 or 7 days post-APV infection, although no signs of swollen heads or sinuses have been observed (1,28,30,42). Other organisms examined during coinfection studies include *Bordetella avium, Pasteurella*-like organisms, *Mycoplasma synoviae, Mycoplasma gallisepticum, Mycoplasma imitans,* infectious bronchitis virus, and turkey herpesvirus (13,15,20,26,31,43). Results from these studies indicated that many agents may be involved in the disease process, although severe disease characterized by swollen sinuses and a swollen head appearance was not typically reproduced experimentally.

In naturally occurring APV infections in turkeys, concurrent infection with Newcastle disease virus (NDV) from either vaccination or field outbreaks contributes to the severity of clinical disease (Dr. Hugo Medina, pers. comm.). The effect of NDV on APV infection has not been determined. Mesogenic and lentogenic strains of NDV are endemic in wild birds, circulate in many poultry populations, and are thought to impair clearance of other respiratory pathogens (9,18,19). Lentogenic NDV vaccine strains may result in tracheal lesions that are postulated to decrease mucociliary clearance, leading to decreased resistance to environmental pathogens and depressed phagocytosis by respiratory macrophages (18,19). The presence of NDV from naturally circulating or vaccine strains in a turkey population makes NDV a potential secondary pathogen in APV outbreaks.

Experimental APV infections are typically less severe than those observed in field cases. Severe APV infections in the field are often concurrent with live NDV vaccination of turkeys, suggesting a role for lentogenic NDV in the APV disease process. To test the role of secondary agents in exacerbating APV infection, we compared infection with APV alone with the coinfection with either a lentogenic strain of NDV or an intermediately pathogenic *E. coli* isolate. Poults receiving dual infections were compared with negative control poults and poults receiving single NDV, APV, and *E. coli* infections for clinical signs and virus isolation from oral swabs and tissues.

MATERIALS AND METHODS

Viruses. The APV isolate used in this study, APV Colorado, was obtained from the National Veterinary Services Laboratories, Ames, IA (courtesy of Dennis Senne). The virus was isolated from commercial turkeys in Colorado having clinical respiratory disease (32). Upon receipt, the isolate was passaged through Vero cells, and the titer was determined by the 50% tissue culture infective dose ($TCID_{50}$) (35).

The lentogenic NDV isolate, TK/VA/695/85 (obtained from D. J. King, Southeast Poultry Research Laboratory, Athens, GA), was isolated from turkey flocks in Virginia in 1985. The virus has an intracerebral pathogenicity index of 0.49, an intravenous pathogenicity index of 0.00, and deduced amino acid sequence similar to the La Sota strain of NDV (27). The virus titer was determined as 50% egg lethal dose in specific-pathogen-free chicken embryos.

Bacteria. *Escherichia coli* O35 (obtained from J. K. Rosenberger, University of Delaware, Newark, DE) was isolated from a broiler flock (37). The bacteria were cultured overnight on MacConkey agar at 37 C. One colony was transferred to brain–heart infusion medium and cultured overnight at 37 C with shaking (37). The bacterial culture was titered through dilutions and plating on MacConkey agar plates 1 day prior to inoculation. This isolate has been characterized as intermediately pathogenic on the basis of its ability to cause 75%–100% mortality with lesions in 1-day-old chickens; however, 14-day-old chickens are relatively resistant, as previously described (36).

Turkeys. Conventional 1-day old turkey poults (British United Turkeys of America, Lewisburg, WV) free from maternal antibodies to APV and NDV were housed at Southeast Poultry Research Laboratory in isolation cabinets under negative pressure within a biosafety level 3 agriculture facility. Poults were provided free access to food and water (5).

Experimental design. *Experiment 1.* Two-week-old turkeys were distributed into six groups of 12 birds each. Turkey poults were numbered prior to inoculation and were euthanatized numerically to induce randomization in the sampling procedure. Group 1 (negative control) received 0.2 ml of phosphate-buffered saline (PBS) via an intranasal (i.n.) inoculation on days 0 and 3. Group 2 (*E. coli*) received 10^7 colony-forming units (CFU) of *E. coli* i.n. in a 0.1-ml dose on day 3. Group 3 (NDV) received 10^5 50% embryo infective dose (EID_{50}) of NDV i.n. in a 0.1-ml dose on day 3. Group 4 (APV) received $10^{4.5}$ $TCID_{50}$ of APV i.n. in a 0.2-ml dose on day 0. Group 5 (APV/*E. coli*) received $10^{4.5}$ $TCID_{50}$ of APV i.n. in a 0.2-ml dose on day 0 and 10^7 CFU of *E. coli* i.n. in a 0.1-ml dose on day 3. Group 6 (APV/NDV) received $10^{4.5}$ $TCID_{50}$ i.n. of APV in a 0.2-ml dose at day 0 and 10^5 EID_{50} of NDV i.n. in a 0.1-ml dose on day 3. The turkey poults were monitored daily for clinical signs. The turkey poults were orally swabbed on 2, 4, 6, 8, 10, and 14 days postinoculation (DPI) with APV. Oral swabs were placed in 1.0 ml of PBS (Invitrogen, Carlsbad, CA) containing antibiotics (1000 units/ml of penicillin, 10 μg/ml of gentamicin, and 5 μg/ml of amphotericin B) (Sigma, St. Louis, MO). On 2, 4, 6, and 8 DPI, two poults from each group were euthanatized by intravenous injection of sodium pentobarbital (100 mg/kg), and tissues were collected for histologic examination and virus isolation. On day 14, the four remaining turkeys were euthanatized and tissues were collected. Lung and trachea were collected under aseptic conditions, ground with mortar and pestle, and prepared with a final suspension of 20% (w/v) in PBS with antibiotics. Serum samples were collected at 14 DPI. All serum, swab, and tissues samples were tested individually.

Experiment 2: Two-week-old turkeys were distributed into four groups with five birds per group in an attempt to reproduce clinical signs observed in Experiment 1. Group 1 (negative control) received 0.2 ml of PBS i.n. on days 0 and 3. Group 2 (NDV) received 0.1 ml of NDV i.n. for a final dose of 10^5 EID_{50} on day 3. Group 3 (APV) received 0.2 ml i.n. of APV for a final dose of $10^{4.5}$ $TCID_{50}$ on day 0. Group 4 (APV/NDV) received $10^{4.5}$ $TCID_{50}$ i.n. of APV in a 0.2-ml dose at day 0 and 10^5 EID_{50} of NDV i.n. in a 0.1-ml dose on day 3. The turkey poults were monitored daily for clinical signs. No oral swabs or tissues samples were collected for virus isolation. Serum samples were collected at 14 DPI.

Virus isolation. Vero cells (ATCC CCL-81) were inoculated with 200 μl of oral swab, trachea, or lung samples and incubated for 45 min, and the medium was replaced (21). Cells were observed daily for cytopathic effect (CPE), characterized by scattered focal areas of cell rounding and syncytia formation (3,11,14). At 7 DPI, cells were subjected to three rounds of freeze thawing and 200 μl of fluid passed onto fresh Vero cells. All samples were passed through Vero cells three times. Virus isolation results were verified by APV and NDV reverse transcriptase (RT)–polymerase chain reaction (PCR).

RNA extraction. RNA was extracted from experimental samples with TRIZOL® reagent (Invitrogen). Vero cells inoculated with experimental samples were subjected to freeze thawing and 200 μl of material added to 1 ml of TRIZOL®. RNA was extracted by following the manufacturer's protocol, resuspended in 30 μl of RNase-free water, and stored at −70 C.

RT-PCR. RT-PCR was used to confirm virus isolation results as well as to distinguish between the presence of NDV or APV in experimental samples. APV primers were developed to detect viruses isolated in the United States (38). The avian pneumovirus primers, APVMLT95 5′-CTGCCTGCAAGGTTAACAGT-3′

and APVMLT745 5′-GTGGCTCCAGTTCCTGCAGA-3′, amplified a 650-base pair (bp) product of the matrix gene of APV. NDV primers to the nucleoprotein gene were included to perform a multiplex RT-PCR as previously described (4). The NDV primers amplify a 309-bp product. RT-PCR was carried out with the One-Step RT-PCR kit (Qiagen, Valencia, CA). Five microliters of sample RNA was added to the RT-PCR mixture with 10 units of ribonuclease inhibitor (Invitrogen) and 0.6 μM of each primer in a final volume of 25 μl. The RT reaction was carried out at 50 C for 30 min with a 15-min initial PCR activation step at 95 C. PCR parameters consist of 94 C for 3 min, 30 cycles of 94 C for 45 sec, 55 C for 30 sec, 72 C for 1 min, followed by an additional extension step of 72 C for 7 min. An additional PCR reaction was included to amplify low levels of viral nucleic acid not detected with the initial RT-PCR reaction (7). For the second PCR reaction, 5 μl of the first reaction was added to a *Taq* PCR Master Mix (Qiagen) along with 0.5 μM of each primer in a total volume of 25 μl. RNA extracted from viral stocks was used as a positive control, and no template and no RT reactions served as negative controls. RT-PCR products were visualized by ethidium bromide staining of 1% Tris-borate EDTA electrophoresis buffer (0.045 M Tris-borate and 0.001 M EDTA) agarose gels.

Histology. Tissues taken for histopathology included upper and lower trachea, lung, heart, air sac, bursa of Fabricius, nasal cavity, lower eyelid, spleen, thymus, thyroid, Harderian gland, and lacrimal gland. The tissues were fixed in 10% buffered formalin solution, sectioned, and stained with hematoxylin and eosin.

Serology. APV antibodies were detected by an indirect enzyme-linked immunosorbent assay (ELISA) specific for APV Colorado. APV Colorado was sucrose purified and coated onto ELISA plates. The antigen was purified as previously described (22). Plates were blocked by the addition of 1% polyvinylpyrrolidone (Sigma). The turkey serum was heat inactivated (37 C for 30 min) and tested at fivefold dilutions from 1/25 to 1/15,625. A horseradish peroxidase–conjugated goat anti-turkey immunoglobulin (Ig)G antibody (Southern Biotech, Birmingham, AL) was added to the ELISA for detection, and o-phenylenediamine dihydrochloride (Sigma) served as substrate. Substrate development was stopped with sulfuric acid by following the manufacturer's protocol, and the optical density (OD) was determined at 490 nm. Turkey serum samples were considered positive if their OD reading was 3 SD above the mean of the negative control sera as previously described (8).

Serum titers to NDV were determined by hemagglutination-inhibition (HI) tests at 14 DPI. Test sera were diluted twofold in PBS, 4 hemagglutination (HA) U of antigen (TK/VA/695/85) was added, and the mixture was incubated at room temperature for 30 min. An equal volume of 0.5% chicken red blood cells in PBS was added. The HI endpoint was determined as the last dilution with complete inhibition of HA activity (27).

Statistics. Antibody titers were analyzed by nonparametric analysis of variance test (Kruskal–Wallis), and, for significantly different groups ($P < 0.05$), Student–Neuman–Keuls multiple comparison test was performed with PC-based software (SigmaStat, Jandel Scientific, San Rafael, CA).

RESULTS

Clinical signs. No clinical signs were observed in the negative control, NDV-, or *E. coli*-inoculated groups throughout Experiment 1. The APV-inoculated group had increased mucus in the nasal cavity as compared with negative control poults on 6 and 8 DPI. At day 4, one poult in the APV/*E. coli* group was gasping for breath and was noticeably depressed. This poult died at day 5 with lesions compatible with septicemia and ventricular dilation. The remainder of the APV/*E. coli*-infected poults were mildly depressed at days 8–10 postinoculation but recovered by day 14. Feeders for the APV/NDV group required less feed than those of other groups, indicating decreased food consumption for the group from day 8 to the end of the experiment. At day 10, three of the remaining four poults in the APV/NDV group had noticeable nasal exudates with infraorbital, periocular, and submandibular swelling that were evident to the end of the experiment (Fig. 1).

In Experiment 2, no clinical signs were observed in the negative control or NDV-inoculated poults. The APV group was mildly depressed at days 4–6 postinoculation. The poults in the APV/NDV group had reduced food consumption and were lethargic by day 10 through the remainder of the experiment. Four of the five birds had infraorbital, periocular, and submandibular swelling noted at day 11 through the end of the experiment.

Virus isolation and RT-PCR. CPE in cell culture was detected from lung, trachea, and swab samples on all days sampled in all groups receiving NDV or APV. Multiplex RT-PCR resulted in the expected 650-bp product for APV and the expected 309-bp product for NDV (Fig. 2). No APV nucleic acid was detected

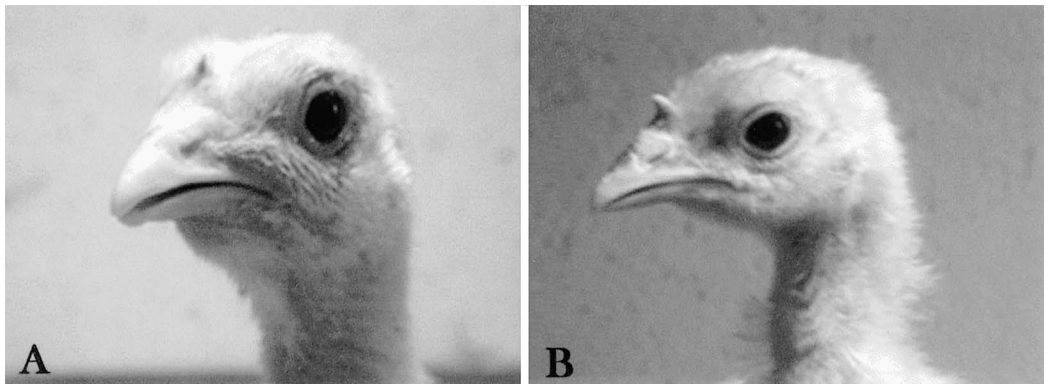

Fig. 1. Turkey poults at 10 days post-APV inoculation. (A) Poults inoculated with APV/NDV with infraobital swelling. (B) Negative control poult.

from the negative control or *E. coli*-inoculated poults at any sample time (Table 1). APV was identified in oral swabs in the APV-inoculated group at days 3, 6, 8, and 10 postchallenge, with positive trachea and lung samples at days 2, 4, 6, and 14. The APV/*E. coli*-inoculated group was positive for APV early in infection, on days 2 and 4 postinoculation. APV was detected throughout the experiment in the group that received APV/NDV (Table 1).

NDV nucleic acid was not detected by RT-PCR in the negative control, *E. coli,* APV, or APV/*E. coli* group (Table 2). NDV was detected only from oral swabs at day 6 and in lungs on days 4, 6, and 14 from poults in the NDV group. In the APV/NDV dual infection group, NDV was detected on days 4, 6, and 8 from oral swabs and days 6, 8, and 14 from lung and trachea.

Histopathology. Inoculation of poults with *E. coli,* NDV, or APV produced only mild lesions in the respiratory tract. Poults inoculated with *E. coli* alone had mild lymphoplasmacytic hyperplasia of the nasal lymphoid tissue and a mild increase in lumenal mucus. These lesions were observed primarily at 8 and 14 DPI. Conversely, poults inoculated with NDV initially developed a mild necrotizing to necropurulent rhinitis, which eventually matured to lymphoplasmacytic in nature and was accompanied by mild epithelial hyperplasia by 8 DPI. In addition, the NDV poults also developed a mild

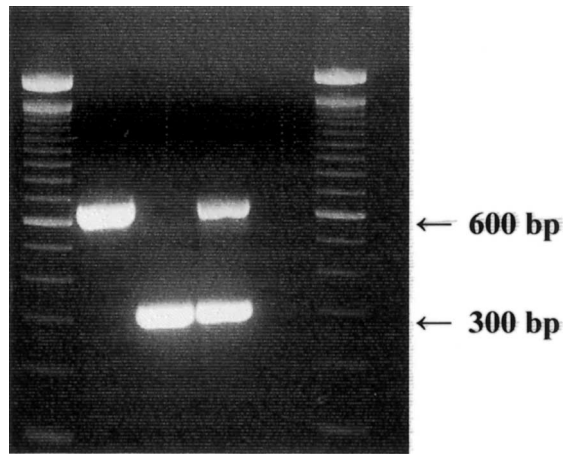

Fig. 2. Multiplex RT-PCR demonstrating the ability to detect both APV and NDV in one sample, representative of experimental data. Lane 1, molecular weight marker; lane 2, APV; lane 3, NDV; lane 4, APV + NDV; lane 5, negative control; lane 6, molecular weight marker.

Table 1. RT-PCR results for APV. Time points are listed as days post-APV inoculation (DPI). NDV and *Escherichia coli* were inoculated at 3 DPI.

Group	Inoculum	Sample[A]	RT-PCR results[B] 2 DPI	4 DPI	6 DPI	8 DPI	10 DPI	14 DPI
1	Control	S	−	−	−	−	−	−
		T	−	−	−	−	NT[C]	−
		L	−	−	−	−	NT	−
2	*E. coli*	S	−	−	−	−	−	−
		T	−	−	−	−	NT	−
		L	−	−	−	−	NT	−
3	NDV	S	−	−	−	−	−	−
		T	−	−	−	−	NT	−
		L	−	−	−	−	NT	−
4	APV	S	+++	−	+++	++	++	−
		T	+	+	−	−	NT	+
		L	++	−	+	−	NT	−
5	APV/*E. coli*	S	+	+	−[D]	−	−	−
		T	+	−	−	−	NT	−
		L	−	+	−	−	NT	−
6	APV/NDV	S	++++	+++	+	−	−	−
		T	++	+	−	−	NT	+
		L	++	+	−	−	NT	+

[A]S = oral swab (n = 10 for 2 and 4 DPI, n = 8 at 6 DPI, N = 4 for 10 and 14 DPI); T = trachea (n = 2); L = lung (n = 2).

[B]− = negative; + = one positive; ++ = two positive; +++ = three positive; ++++ = four positive samples.

[C]NT = not tested.

[D]One mortality at 5 DPI.

lymphoplasmacytic airsacculitis that was observed at 8 and 14 DPI. Lesions in the nasal cavity of poults inoculated with APV alone consisted of only mild lymphoplasmacytic inflammation at 8 and 14 DPI. However, APV also induced mild heterophilic conjunctivitis with edema in poults sampled at 4 and 6 DPI. Mild airsacculitis also was observed in both APV-inoculated poults sampled at 6 DPI.

More pronounced lesions were observed in poults inoculated with either APV/NDV or APV/*E. coli.* Inoculation of poults with APV/*E. coli* resulted in a heterophilic to lymphoplasmacytic rhinitis and sinusitis with epithelial hyperplasia; the severity of these lesions varied from mild to marked. In addition, mild conjunctivitis was observed in one poult collected at 4 and 6 DPI. The inoculation of poults with APV/NDV produced the most consistent and severe lesions in the nasal cavity and sinuses, air sacs, and conjunctiva. Lesions in the nasal cavity and sinus consisted of moderate to severe mixed heterophilic to mononuclear inflammation with epithelial hyperplasia and an accumulation of mucus within the cavity. Lesions in the air sacs and conjunctiva in APV/NDV-inoculated poults were typically mild to moderate and morphologically resembled those previously described.

Serology. Negative control, *E. coli*-, and NDV-inoculated groups had no detectable antibodies to APV at 14 DPI. All three groups that received APV had detectable antibody titers at day 14 postinfection (Fig. 3). No significant differences were detected in the titers to APV among the three APV-inoculated groups in either Experiment 1 or 2.

No NDV titers were detected in the negative control, APV/*E. coli, E. coli,* or APV groups. Both the NDV and APV/NDV groups had HI titers to NDV at 14 days postinfection (Fig. 4). No significant differences were detected in the titers to NDV between the two NDV-inoculated groups in either Experiment 1 or 2.

DISCUSSION

Natural infections with APV are more severe than experimental infections, suggesting that secondary agents or other factors play a prom-

Table 2. RT-PCR results for NDV. Time points are listed as days post-APV inoculation (DPI). NDV and *Escherichia coli* were inoculated at 3 DPI.

			RT-PCR results[B]					
Group	Inoculum	Sample[A]	2 DPI	4 DPI	6 DPI	8 DPI	10 DPI	14 DPI
1	Control	S	−	−	−	−	−	−
		T	−	−	−	−	NT[C]	−
		L	−	−	−	−	NT	−
2	*E. coli*	S	−	−	−	−	−	−
		T	−	−	−	−	NT	−
		L	−	−	−	−	NT	−
3	NDV	S	−	−	+++	−	−	−
		T	−	+	−	−	NT	−
		L	−	−	+	−	NT	+
4	APV	S	−	−	−	−	−	−
		T	−	−	−	−	NT	−
		L	−	−	−	−	NT	−
5	APV/*E. coli*	S	−	−	−[D]	−	−	−
		T	−	−	−	−	NT	−
		L	−	−	−	−	NT	−
6	APV/NDV	S	−	++	+	+	−	−
		T	−	−	−	+	NT	−
		L	−	−	+	−	NT	+

[A]S = oral swab (n = 10 for 2 and 4 DPI, n = 8 at 6 DPI, N = 4 for 10 and 14 DPI); T = trachea (n = 2); L = lung (n = 2).
[B]− = negative; + = one positive; ++ = two positive; +++ = three positive samples.
[C]NT = not tested.
[D]One mortality at 5 DPI.

inent role in the clinical disease process. Many studies have focused on the effects of bacteria and viruses on APV infection with various degrees of success at reproducing the clinical signs seen in the field (13,15,20,26,28,31,43). Experimentally, APV infections are similar in both chickens and turkeys, although some factors appears to affect host susceptibility (10). Experimental infections with type C APV isolates have resulted in various degrees of clinical signs, with the Colorado isolate resulting in milder clinical signs and microscopic lesions than the APV Minnesota 2A isolate (14,23,32). This study demonstrated that coinfection with APV/

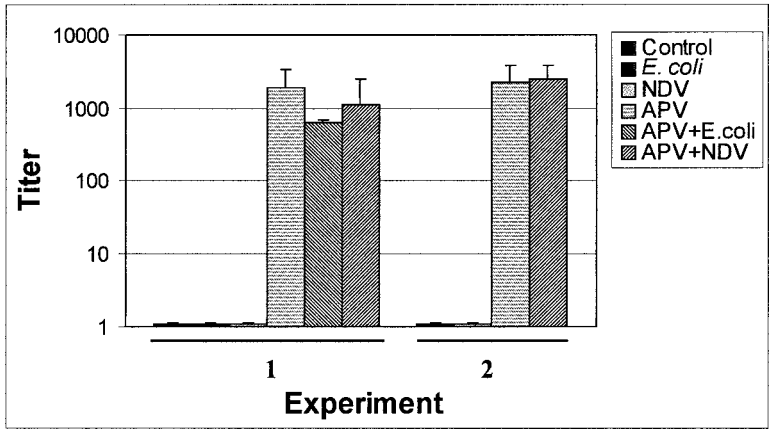

Fig. 3. Serologic response against APV in turkeys at 14 days postinoculation determined by an indirect ELISA.

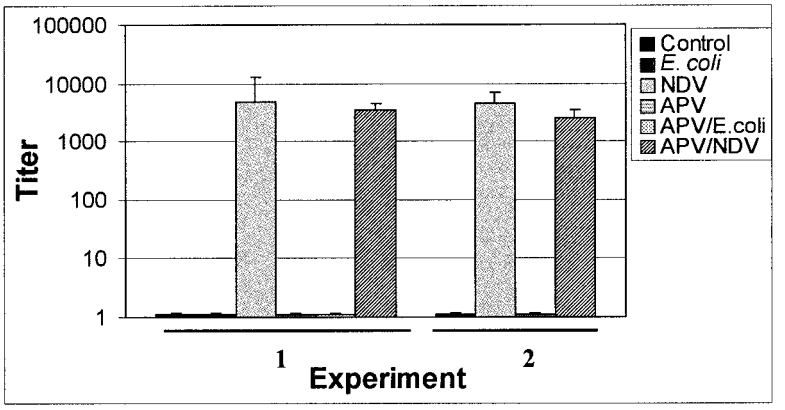

Fig. 4. Serologic response against NDV infection in turkeys at 14 days postinoculation determined by HI titers.

NDV resulted in reproducible clinical signs comparable with descriptions of APV type C field infections, including infraorbital, periocular, and submandibular swelling (16). These clinical signs were not observed in the poults receiving APV alone or in the negative control, *E. coli,* or NDV groups. Poults receiving APV/*E. coli* exhibited mild clinical signs early during infection, but no swelling of sinuses was observed, as previously described (13,27). This finding supports the hypothesis that APV infection is exacerbated by dual infections and suggests lentogenic NDV may play a role in the more severe field cases of APV infections.

The gross and histologic lesions in the APV/NDV-inoculated poults most closely paralleled those previously reported in turkey flocks naturally infected with APV (12). Poults inoculated with NDV developed histologic lesions in the nasal cavity and air sacs analogous to those previously described in field cases of lentogenic NDV, including deciliation to epithelial necrosis and edema followed by a lymphoplasmacytic inflammation and epithelial hyperplasia (2,17). The histologic lesions in the upper respiratory tract in the APV group were only minor in comparison with those previously reported for field cases of APV infection (23). Gross lesions of turkeys observed during an APV infection reported from field cases have included the accumulation of mucus in the nares and infraorbital sinuses accompanied by cloudiness and thickening of the air sacs, pericardial sac, and peritoneum (23). Histologic changes in the nares and trachea occurring with natural APV infection have consisted of an early loss of cilia followed by submucosal edema and hyperemia, heterophilic infiltrates with exocytosis, and an accumulation of inflammatory exudates within the lumen (7,16,25). After the acute infection, the cellular inflammation matures from heterophilic to mononuclear (25). Only the poults inoculated with APV/NDV in this investigation had gross and histologic lesions of comparable distribution and severity with those previously reported with spontaneous APV infection. This finding again suggests a synergistic relationship between APV and other respiratory pathogens such as NDV.

No difference in serologic responses to APV and NDV were noted in the single or dual infection groups. This finding differs from those in previous experiments where Ganapathy *et al.* (20) showed that higher serum ELISA titers for humoral response were noted in dual infection with APV and *Mycoplasma imitans* as compared with single infection of APV. Because APV generally results in a localized infection of the upper respiratory tract, systemic antibodies may not be the optimal parameter to measure protection or exposure. The ELISA used in these experiments tests only for levels of IgG, whereas measuring local IgA levels may help monitor response to local respiratory infections more efficiently.

APV can generally be detected by virus isolation from 3 to 5 DPI in nasal and sinus tissues (14). In this study, we used RT-PCR to confirm the virus isolation results, differentiate between APV and NDV infections, and extend

the detection time as described (6). In the current study, the majority of virus isolation and RT-PCR–positive results in the APV group occurred early during infection. The poults in the APV/NDV group had longer periods of virus recovery, and a higher percentage of birds were positive at each sampling. The increased severity of clinical signs as well as the increase in duration and number of poults shedding virus indicates that dual infection of APV and NDV resulted in disease more closely resembling field outbreaks. This finding supports the hypothesis that the APV disease pattern in the field may be linked to concurrent NDV infections and potentially even live NDV vaccines.

Cook *et al.* (15) have shown that vaccination with live infectious bronchitis virus (IBV) vaccine and concurrent infection with a pathogenic type B APV isolate in chickens results in reduced APV recovery and antibody production. Investigators that used IBV and NDV demonstrated that competition or interference occurs when two viruses are known to replicate in the same tissue (15). The competition for the replication site and the knowledge that APV replicates slower in tissue culture than does IBV may explain the apparent decrease in APV virus recovery and subsequent antibody production (15). This situation does not appear to have occurred in the present experiment with APV and NDV in turkeys. The differences in the IBV and NDV experiments may be due to host response, differences in inoculation schemes, or the interaction between APV/NDV or that of APV/IBV. Although NDV also replicates more quickly than APV in cell culture systems, the inoculation of APV 3 days prior to NDV appears to have allowed the APV time to infect epithelial cells but did not block the ability of NDV to establish an infection. This is demonstrated by the similar rates of virus recovery and antibody detection in the groups that received single or dual APV and NDV inoculations. Instead, a synergistic effect appears to occur with increased virus recovery detected at each time point as well as increased length of viral shed. Dual infection with APV and *E. coli* caused mild morbidity in the turkey poults on days 8–10 post-APV inoculation; this was also evident in the increased lesions noted upon necropsy and histologic examination. *Escherichia coli* inoculation appears to have decreased APV replication as noted by decreased APV detection in the APV/*E. coli* group compared with the APV alone group. Further studies are needed to completely understand the kinetics and synergistic effects of APV and NDV described in this study.

The results of these experiments demonstrate that dual infection with APV and NDV produces what has been diagnosed as clinical APV. The correlation between increased clinical signs and coinfection with a lentogenic NDV isolate suggests a need for better monitoring of NDV vaccination programs. Reports of some field outbreaks of APV have been associated with increased respiratory disease associated with live NDV vaccination (H. Medina and M. Kumar, pers. comm.). The vaccination of a turkey flock with an underlying APV infection may result in an increase in both NDV and APV disease. Care should be taken to control or prevent APV infections prior to NDV vaccinations. Increased biosecurity and restricted movement of people and material between APV-infected and noninfected farms is needed to prevent and control APV infections (45).

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ACKNOWLEDGMENTS

We acknowledge Joan Beck, Roger Brock, Jack King, Bruce Seal, and Darrell Kapczynski for assistance and scientific advisement. This research was supported by USDA/ARS CRIS project 6612-32000-022.